Original article

MOLECULAR METABOLISM

Glut4 expression defines an insulin-sensitive hypothalamic neuronal population

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ABSTRACT

Insulin signaling in the CNS modulates satiety and glucose metabolism, but insulin target neurons are poorly defined. We have previously shown that ablation of insulin receptors (InsR) in Glut4-expressing tissues results in systemic abnormalities of insulin action. We propose that Glut4 neurons constitute an insulin-sensitive neuronal subset. We determined their gene expression profiles using flow-sorted hypothalamic Glut4 neurons. Gene ontology analyses demonstrated that Glut4 neurons are enriched in olfacto-sensory receptors, M2 acetylcholine receptors, and pathways required for the acquisition of insulin sensitivity. Following genetic ablation of InsR, transcriptome profiling of Glut4 neurons demonstrated impairment of the insulin, peptide hormone, and cAMP signaling pathways, with a striking upregulation of anion homeostasis pathway. Accordingly, hypothalamic InsR-deficient Glut4 neurons showed reduced firing activity. The molecular signature of Glut4 neurons is consistent with a role for this neural population in the integration of olfacto-sensory cues with hormone signaling to regulate peripheral metabolism.

Keywords Insulin signaling; CNS; Glut4 neurons; Neurotransmitter receptor; Ion channel

1. INTRODUCTION

Insulin acts through the insulin receptor (InsR) in the CNS to regulate food intake [1], counterregulatory response to hypoglycemia [2], and leptin sensitivity [3]. However, anatomic and functional mapping of insulin's CNS action is not nearly as detailed as those of other hormones [4]. This is partly due to the fact that InsR are thought to be widespread among neurons, not to mention glial, and non-neural cells [5]. Glucose utilization is thought to occur largely in an insulin-independent manner in the CNS [6]. Nonetheless, a sub-population of widely scattered neurons possesses the insulin-responsive glucose transporter, Glut4 [7]. While the function of this transporter in glucose metabolism continues to be a matter of speculation, it's a fact that removing InsR from Glut4 neurons has a profound detrimental effect on peripheral metabolism. Thus mice lacking InsR in Glut4-expressing tissues develop type 2 diabetes [7], highlighting the contribution of CNS insulin resistance to the pathophysiology of diabetes [8].

In this study, we set out to investigate the nature of Glut4 neurons in the hypothalamus, a key regulatory site for appetite and glucose homeostasis [9]. As a first step toward characterizing the physiological function(s) of Glut4 neurons, we determined their gene expression profiles. To this end, we generated transgenic mice with chemically defined Glut4 neurons [7]. We flow-sorted Glut4 neurons and compared their gene expression profile with that of non-Glut4 neurons using a previously reported protocol [10]. Interestingly, we found pathways critical for sensory perception, receptor signaling, and response to nutrient are significantly enriched in the Glut4 neurons, consistent with the hypothesis that Glut4 neurons have critical roles in metabolic regulation.

2. MATERIAL AND METHOD

2.1. Mice

Gt(Rosa)26Sor$^{tm9(CAG\text{-}tdTomato)Hze}$ mice were from the Jackson Laboratories. GIRKO mice were generated as previously described [7]. The Columbia University Animal Care and Utilization Committee approved all procedures. Normal chow diet (NCD) had 62.1% calories from carbohydrates, 24.6% from protein and 13.2% from fat (PicoLab rodent diet 20, 5053; Purina Mills).

2.2. Flow cytometry, gene profiling, and quantification of neurons

We dissociated mediobasal hypothalami from 3-week-old mice with transgenes (*Glut4-Cre* [line 535]; *Rosa-Tomato*; *InsR lox* allele) with papain dissociation kit (Worthington Biochemical). We gated live neurons to collect Rfp-positive neurons. We performed microarray for gene profiling using a procedure described previously [10]. We used Partek Genomic Suite and R for pathway analysis and heatmap generation. We analyzed the FACS data using Flowjo software. We quantified immunofluorescence using Image J software.

2.3. Electrophysiological studies

For patch-clamp recording in acute slices, we sectioned 350-μm-thickn coronal brain sections in a cold sucrose-based cutting solution containing (in mM): 195 sucrose, 10 NaCl, 25 $NaHCO_3$, 25 glucose, 2.5 KCl, 1.25 NaH_2PO_4, 2 Na pyruvate, 0.5 $CaCl_2$, and 7 $MgCl_2$ (pH 7.3)

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using a vibratome (Leica VT1000S). Slices were incubated in a submerged chamber at room temperature for at least 90 min before recordings, perfused with recording solution containing (in mM): 125 NaCl, 2.5 KCl, 1.25 NaH_2PO_4, 25 $NaHCO_3$, 2 $CaCl_2$, 1 $MgCl_2$, 25 glucose (pH 7.3) and continuously bubbled with 95% O_2 and 5% CO_2. We selected cells using an upright microscope fitted with fluorescence optics (Nikon) and patched under IR-DIC optics. We acquired patch-clamp recordings with a MultiClamp 700B amplifier and Clampex data acquisition software (Axon Instruments) at room temperature. We digitized data at 10 kHz with a 2 kHz low-pass filter. We fabricated a glass pipette of 5–8 MΩ and filled it with electrode solution (in mM): 130 K gluconate, 10 KCl, 5 HEPES, 5 $MgCl_2$, 0.06 $CaCl_2$, 0.6 EGTA, 2 MgATP, 0.2 Na_2GTP, and 20 Phosphocreatine. We adjusted osmolarity to 310 mOsm and pH to 7.3 with KOH. We assessed electrical access in the whole-cell recording mode using a small-voltage step pulse before and at the end of the current-clamp recordings. We discarded recordings in which the series resistance had been >30 MΩ. Resting membrane potential and spontaneous action potentials were recorded in the current-clamp mode with no current injection. Detection and measurements of action potentials were performed using MiniAnalysis software (Synaptosoft).

2.4. Statistical analyses

We analyzed data with Student's *t*-test or 1way or 2way ANOVA using GraphPad Prism and Partek Genomic Suite software. We used the customary threshold of $p < 0.05$ to declare statistical significance.

3. RESULTS

3.1. Glut4 neurons are enriched in olfacto-sensory receptors and hormone signaling pathways

We took an unbiased approach to characterize Glut4 neurons. We generated *Glut4-Cre: Rosa26-Tomato* mice, in which Glut4 neurons were labeled by Cre-mediated activation of red fluorescent protein (Rfp). We used fluorescence-activated cell sorting (FACS) to collect Rfp-labeled Glut4 neurons and non-fluorescent control neurons from basal hypothalami of transgenic mice. We compared transcriptomes of Glut4 and control neurons by microarray analysis. Gene ontology (GO) enrichment analysis demonstrated that the Glut4 neuron transcriptome was enriched in olfacto-sensory systems and G-protein coupled receptors. Other classes of enriched genes could be subsumed under these classifications. In addition, we found enrichment in genes required for nutrient sensing and glucose utilization, consistent with the notion that these neurons have metabolic control activity (Table 1). Indeed, GO ANOVA analysis indicated an overrepresentation of genes involved in the maturation of metabolically active cells (adipocytes). This class included transcriptional regulators such as Ppar-γ, Tcf7l2, and others that could be subsumed under the classification of histone modification genes, which was also enriched in Glut4 neurons. GO pathway analysis revealed enrichment in genes required for O-glycan biosynthesis and ribosome biogenesis (Table 1).

In an attempt to assign Glut4 neurons to a specific class of neurons, we carried out a separate analysis of genes that are essential for neuronal activity, including neurotransmitter receptors and ion channels. A heatmap rendering shows that most neurotransmitter receptors have variable levels of enrichment in Glut4 neurons (Figure 1A). We analyzed the data by grouping the major sub-classes of neurotransmitter receptors (Figure 1B–I). Overall, we found no enrichment of specific neurotransmitter subsets, indicating that Glut4 neurons likely include different neuronal subtypes (Figure 1B–I). However, we found a significant enrichment of the M2 subtype muscarinic receptor, along with a less impressive increase of dopamine D2 receptor (Figure 1G). The expression of ion channels showed no particular pattern of enrichment in Glut4 neurons (Figure 1J). We analyzed the expression of major sub-classes of ion channels (Figure 1K–N), but they revealed no significant change.

Gene ontology enrichment

Function	Enrichment score	*p*-Value	%Genes in group represented
Olfactory receptor activity	17.3227	3.00E-08	4.07031
Sensory perception of smell	16.485	6.93E-08	3.95328
Sensory perception of chemical stimulus	15.3567	2.14E-07	3.75313
Sensory perception	11.6154	9.03E-06	3.20382
G-protein coupled receptor activity	10.9879	1.69E-05	3.00578
Neurological system process	10.0768	4.20E-05	2.94638
Transmembrane signaling receptor activity	9.06199	0.000115992	2.74177
G-protein coupled receptor signaling pathway	8.72103	0.000163119	2.7376
Signaling receptor activity	7.98683	0.000339911	2.62781
Cellular response to nutrient	7.31929	0.000662631	27.2727
Regulation of transcription by glucose	5.55087	0.00388407	33.3333

Gene ontology ANOVA

GO description	# of probes	GO ID	*p*-Value (attribute)
Fat cell differentiation	63	45444	0.00140471
Phospholipid homeostasis	8	55091	0.00212119
Sulfonylurea receptor binding	2	17098	0.00325252
Calcium- and calmodulin-responsive adenylate cyclase activity	5	8294	0.00384257
Positive regulation of histone modification	41	31058	0.00548655
Negative regulation of small GTPase mediated signal transduction	30	51058	0.00609217
Mitochondrial respiratory chain	5	5746	0.00635338
Pyrimidine ribonucleoside binding	4	32551	0.00722503
Aging	76	7568	0.00741073
Carnitine transport	4	15879	0.00883626

Pathway enrichment

Pathway name	# of probesets	Pathway ID	*p*-Value (attribute)
Mucin type O-Glycan biosynthesis	25	Kegg pathway 48	0.00864224
Ribosome biogenesis in eukaryotes	75	Kegg pathway 100	0.0275843
Protein export	19	Kegg pathway 22	0.0642122
Regulation of autophagy	28	Kegg pathway 9	0.0965468

Table 1: Gene ontology and pathway analysis of Glut4 neurons.
Top categories in each group of gene ontology analyses performed using RNA profiling data from flow-sorted Glut4 neurons.

3.2. Characterization of InsR-deficient Glut4 neurons

Our hypothesis was that Glut4 neurons are important to mediate CNS insulin signaling. To test this possibility, we interrogated the gene expression profiles of InsR-deficient Glut4 neurons using the same method described above (exon microarray hybridization of flow-sorted hypothalamic neurons), and performed electrophysiological studies.

We used Tomato fluorescence to identify Glut4 neurons in WT and GIRKO mice bearing either two/one (*i.e.* $Insr^{lox/+}$ or $Insr^{+/-}$) or no intact *InsR* allele ($Insr^{\Delta/\Delta}$ or $Insr^{\Delta/-}$). We collected Tomato-positive (i.e. Glut4 neurons) and Tomato-negative (i.e. non-Glut4 cells) fractions from both WT and GIRKO mice (Figure 2A). Using DNA extracted post-sort from two independent sample sets (Figure 2B), we confirmed that Glut4 neurons from GIRKO mice had minimum intact *InsR* allele. We compared gene expression profiles from sorted neurons (Figure 2C). Gratifyingly, gene set enrichment analysis of the array results showed that *InsR* was one of the most decreased mRNA (Supplemental Table 1), and that impaired expression of genes involved in the cellular responses to insulin topped the list of altered pathways, demonstrating the robustness of the method (Table 2).

